

## Changes in natural killer cells and exhausted memory regulatory T Cells with corticosteroid therapy in acute autoimmune hepatitis

Jeffery, Hannah C.; Braitch, Manjit K.; Bagnall, Chris; Hodson, James; Jeffery, Louisa E.; Wawman, Rebecca E.; Wong, Lin Lee; Birtwistle, Jane; Bartlett, Helen; Lohse, Ansgar W.; Hirschfield, Gideon M.; Dyson, Jessica; Jones, David; Hubscher, Stefan G.; Klenerman, Paul; Adams, David H.; Oo, Ye H.

DOI:

[10.1002/hep4.1163](https://doi.org/10.1002/hep4.1163)

License:

Creative Commons: Attribution (CC BY)

*Document Version*

Publisher's PDF, also known as Version of record

*Citation for published version (Harvard):*

Jeffery, HC, Braitch, MK, Bagnall, C, Hodson, J, Jeffery, LE, Wawman, RE, Wong, LL, Birtwistle, J, Bartlett, H, Lohse, AW, Hirschfield, GM, Dyson, J, Jones, D, Hubscher, SG, Klenerman, P, Adams, DH & Oo, YH 2018, 'Changes in natural killer cells and exhausted memory regulatory T Cells with corticosteroid therapy in acute autoimmune hepatitis', *Hepatology Communications*, vol. 2, no. 4, pp. 421-436.  
<https://doi.org/10.1002/hep4.1163>

[Link to publication on Research at Birmingham portal](#)

### General rights

Unless a licence is specified above, all rights (including copyright and moral rights) in this document are retained by the authors and/or the copyright holders. The express permission of the copyright holder must be obtained for any use of this material other than for purposes permitted by law.

- Users may freely distribute the URL that is used to identify this publication.
- Users may download and/or print one copy of the publication from the University of Birmingham research portal for the purpose of private study or non-commercial research.
- User may use extracts from the document in line with the concept of 'fair dealing' under the Copyright, Designs and Patents Act 1988 (?)
- Users may not further distribute the material nor use it for the purposes of commercial gain.

Where a licence is displayed above, please note the terms and conditions of the licence govern your use of this document.


When citing, please reference the published version.

### Take down policy

While the University of Birmingham exercises care and attention in making items available there are rare occasions when an item has been uploaded in error or has been deemed to be commercially or otherwise sensitive.

If you believe that this is the case for this document, please contact [UBIRA@lists.bham.ac.uk](mailto:UBIRA@lists.bham.ac.uk) providing details and we will remove access to the work immediately and investigate.

# Changes in Natural Killer Cells and Exhausted Memory Regulatory T Cells With Corticosteroid Therapy in Acute Autoimmune Hepatitis

Hannah C. Jeffery,<sup>1</sup> Manjit K. Braitch,<sup>1</sup> Chris Bagnall,<sup>2</sup> James Hodson,<sup>3</sup> Louisa E. Jeffery,<sup>1</sup> Rebecca E. Wawman,<sup>1,4</sup> Lin Lee Wong,<sup>5</sup> Jane Birtwistle,<sup>6</sup> Helen Bartlett,<sup>1</sup> Ansgar W. Lohse,<sup>7</sup> Gideon M. Hirschfield,<sup>1,8</sup> Jessica Dyson,<sup>5</sup> David Jones,<sup>5</sup> Stefan G. Hubscher,<sup>1,9</sup> Paul Klenerman,<sup>10</sup> David H. Adams <sup>1,8</sup> and Ye H. Oo<sup>1,8</sup>

Autoimmune hepatitis (AIH) is an immune-mediated liver disease currently treated by immunosuppressive medications with significant side effects. Thus, novel mechanistic treatments are greatly needed. We performed prospective deep immunophenotyping of blood immune cells in patients with acute AIH before and after corticosteroid therapy. Blood samples from 26 patients with acute AIH (United Kingdom-AIH Consortium) were phenotyped by flow cytometry at baseline and 4 months after starting corticosteroids. Pretreatment liver tissues were stained for forkhead box P3-positive (FOXP3<sup>POS</sup>) regulatory T cells (Tregs), clusters of differentiation (CD)56<sup>POS</sup> natural killer (NK) cells, and chemokine (C-X-C motif) ligand 10. Chemokine secretion by cultured primary hepatocyte and biliary epithelial cells was measured by enzyme-linked immunosorbent assay. Functional coculture assays with stimulated NK cells and Tregs were performed. CD161 ligand, lectin-like transcript-1 expression by intrahepatic immune cells was demonstrated with flow cytometry. Frequencies of NK<sup>bright</sup> cells declined with therapy ( $P < 0.001$ ) and correlated with levels of alanine aminotransferase ( $P = 0.023$ ). The Treg:NK<sup>bright</sup> ratio was lower pretreatment, and Tregs had an activated memory phenotype with high levels of CD39, cytotoxic T lymphocyte antigen 4, and FOXP3 but also high programmed death ligand 1, indicating exhaustion. Coculture experiments suggested the Tregs could not efficiently suppress interferon- $\gamma$  secretion by NK cells. Both Tregs and NK cells had high expression of liver infiltration and T helper 17 plasticity-associated marker CD161 ( $P = 0.04$ ). Pretreatment and CD161<sup>POS</sup> NK cells expressed high levels of perforin and granzyme B, consistent with an activated effector phenotype ( $P < 0.05$ ). Lectin-like transcript 1, a ligand for CD161, is expressed on intrahepatic B cells, monocytes, and neutrophils. **Conclusion:** Activated effector NK cells, which correlate with biochemical measurements of hepatitis, and exhausted memory Tregs are increased in the blood of patients with treatment-naïve AIH and decline with corticosteroid therapy. Inadequate regulation of NK cells by exhausted FOXP3<sup>POS</sup> Tregs may play a role in AIH pathogenesis and contribute to liver injury. (*Hepatology Communications* 2018;2:421-436)

## Introduction

Autoimmune hepatitis (AIH) is an immune-mediated liver disease characterized by interface and lobular hepatitis<sup>(1)</sup> comprising infiltrates of both effector and regulatory T lymphocytes (Tregs).<sup>(1,2)</sup> There have been no new therapies for

AIH for more than 3 decades, and it is becoming increasingly clear that there are limitations to the long-term safety and efficacy of the nonspecific and empirical treatment in current use.<sup>(3)</sup> Thus, there is a need for more effective mechanistically grounded approaches to treatment, and a better understanding of the immune make-up of patients before they receive treatment is

*Abbreviations:* AIH, autoimmune hepatitis; ALT, alanine aminotransferase; CCR7, chemokine (C-C motif) receptor 7; CD, clusters of differentiation; CTLA-4, cytotoxic T lymphocyte antigen 4; CXCL-10, chemokine (C-X-C motif) ligand 10; CXCR3, cysteine-X-cysteine receptor 3; EM, effector memory; FOXP3, forkhead box P3; IFN $\gamma$ , interferon- $\gamma$ ; IgG, immunoglobulin G; IL, interleukin; LIT1, lectin-like transcript 1; NK, natural killer; NKT, natural killer T cells; PD1, programmed death ligand 1; Th, T helper; TNF $\alpha$ , tumor necrosis factor  $\alpha$ ; Treg, regulatory T cell; UK-AIH, United Kingdom Autoimmune Hepatitis; ULN, upper limit of normal.

Received September 25, 2017; accepted January 22, 2018.

Additional Supporting Information may be found at [onlinelibrary.wiley.com/doi/10.1002/hep4.1163/full](https://onlinelibrary.wiley.com/doi/10.1002/hep4.1163/full).

crucial for developing such novel immune cell/pathway-targeted treatments for AIH.

One of the challenges in studying the immune status in patients who are treatment naive is the rapid initial response to corticosteroid treatment. This means that most patients are started on therapy before they can be investigated. In the vast majority of patients, this treatment is with corticosteroid or immunosuppressive therapy, which by nature alters immune activation status. Although studies have been performed to dissect the immune cell composition of patients with AIH on treatment, the immune balance between regulatory and effector cells in the treatment-naive state and during longitudinal follow-up of patients with acute AIH on maintenance immunosuppression is not known.

An imbalance between clusters of differentiation (CD)4<sup>positive[pos]</sup>CD25<sup>pos</sup>CD127<sup>low</sup> Tregs<sup>(4)</sup> and effector T cells has been proposed to contribute to the immune

pathogenesis of AIH.<sup>(2,5-7)</sup> The differentiation and function of Tregs is controlled by transcription factor forkhead box P3 (FOXP3),<sup>(8)</sup> and mutation in FOXP3 leads to a severe multiorgan autoimmune disorder (immunodysregulation polyendocrinopathy enteropathy X-linked syndrome) in humans.<sup>(9)</sup> CD56<sup>pos</sup>CD3<sup>negative[neg]</sup> natural killer (NK) cells are a key component of the innate immune system and are involved in human autoimmune diseases, such as systemic lupus erythematosus<sup>(10)</sup> and rheumatoid arthritis.<sup>(11)</sup> NK cells are abundant in the liver.<sup>(12)</sup> The activation and expansion of NK cells occurs in the early stages of AIH as NK cells function as a first response to liver injury<sup>(13)</sup> and carry out diverse functions, including cytotoxicity, which may be directed at target cells, and cytokines interferon- $\gamma$  (IFN $\gamma$ ) and tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) release, which can promote the maturation of antigen-presenting cells to drive an adaptive immune response.<sup>(14)</sup> Although NK cells have been

*Supported by a Medical Research Council Clinician Scientist Award (G1002552 to Y.H.O.); Queen Elizabeth Hospital Birmingham Charity and National Institute for Health Research (NIHR) Birmingham Biomedical Research Unit (H.C.J.); United Kingdom Autoimmune Hepatitis (UK-AIH) Translational Research Collaboration (M.K.B.); Wellcome Trust (109965MA) and NIHR (P.K.); NIHR Biomedical Research Centre Oxford; NIHR Newcastle Biomedical Research Centre and the NIHR Rare Diseases Translational Research Collaboration (J.K.D.); NIHR Rare Diseases Translational Research Collaboration, United Kingdom (UK-AIH study).*

*This study was conducted on behalf of the UK-AIH Consortium.*

*This paper presents independent research supported by the NIHR Biomedical Research Unit Birmingham and Biomedical Research Centres, Newcastle and Oxford, and the NIHR Rare Diseases Translational Research Collaboration, United Kingdom. The views expressed are those of the authors and not necessarily those of the National Health Service, the NIHR, or the Department of Health.*

*Copyright © 2018 The Authors. Hepatology Communications published by Wiley Periodicals, Inc., on behalf of the American Association for the Study of Liver Diseases. This is an open access article under the terms of the [Creative Commons Attribution](#) License, which permits use and distribution in any medium, provided the original work is properly cited.*

*View this article online at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).*

*DOI 10.1002/hep4.1163*

*Potential conflict of interest: Nothing to report.*

## ARTICLE INFORMATION:

From the <sup>1</sup>Centre for Liver Research, Institute of Immunology and Immunotherapy and National Institute of Health Research Inflammation Biomedical Research Centre Birmingham, University of Birmingham, Birmingham, United Kingdom; <sup>2</sup>Human Biomaterials Resource Centre, University of Birmingham, United Kingdom; <sup>3</sup>Institute of Translational Medicine, University Hospitals Birmingham National Health Services Foundation Trust, University of Birmingham, Birmingham, United Kingdom; <sup>4</sup>School of Life Sciences, Faculty of Health and Life Sciences, Coventry University, Coventry, United Kingdom; <sup>5</sup>Newcastle Biomedical Research Centre and Newcastle University, Newcastle, United Kingdom; <sup>6</sup>Clinical Immunology Department, University of Birmingham, Birmingham, United Kingdom; <sup>7</sup>University of Hamburg, Hamburg, Germany; <sup>8</sup>Liver Transplantation and Hepatobiliary Unit, Queen Elizabeth Hospital, University Hospitals Birmingham National Health Service Foundation Trust, Birmingham, United Kingdom; <sup>9</sup>Department of Histopathology, Queen Elizabeth Hospital, University Hospitals Birmingham National Health Service Foundation Trust, Birmingham, United Kingdom; <sup>10</sup>Peter Medawar Building of Pathogen Research, University of Oxford, Oxford, United Kingdom.

## ADDRESS CORRESPONDENCE AND REPRINT REQUESTS TO:

Ye Htun Oo, M.B.B.S., Ph.D.  
Medical Research Council Clinician Scientist and Consultant  
Hepatologist  
Centre for Liver Research and NIHR Birmingham Biomedical  
Research Unit

University of Birmingham  
Vincent Drive, Edgbaston  
Birmingham, B15 2TT, United Kingdom  
E-mail: [y.h.oo@bham.ac.uk](mailto:y.h.oo@bham.ac.uk)  
Tel: +44 121 415 8700

shown to be particularly important in liver injury in viral hepatitis,<sup>(15,16)</sup> their frequency, function, and interaction with Tregs in the initial presentation of AIH, before corticosteroid therapy, and during longitudinal follow-up is not known. In addition, recruitment and positioning of immune cells from peripheral circulation to the inflamed liver is also crucial for their function.<sup>(2,17)</sup>

We hypothesized that dysregulation of effector innate NK cells and Tregs may play a role in acute AIH and that understanding this balance may help us to develop specific therapies and predict response to treatment. Based on this premise, we carried out a detailed, prospective, longitudinal immunophenotyping of immune cell subsets in patients with acute AIH before and 4 months after corticosteroid therapy and studied the influence of regulatory and effector lymphocytes on NK cells in functional assays. In addition, we screened for the presence of recruitment and positioning signals of liver-infiltrated immune cells in treatment-naïve AIH livers.

## Materials and Methods

### ETHICS STATEMENT

Written informed consent was obtained from all subjects under United Kingdom-AIH (UK-AIH) ethics. Peripheral blood and liver tissues were collected with local research ethics committee approval (Newcastle, 14/LO/0303; Birmingham, CA/5192).

### BLOOD AND LIVER TISSUE

We used 10 mL ethylene diamine tetraacetic acid-chelated peripheral blood samples from patients diagnosed with AIH. AIH was diagnosed according to international AIH scoring criteria,<sup>(18)</sup> which included scores from serologic, virology, and histologic information. Samples were transported overnight to the National Institute for Health Research Biomedical Research Unit, University of Birmingham, for next day analysis of immune phenotype by flow cytometry.

Pretreatment, paraffin-fixed, liver biopsy sections were obtained from 6 Birmingham patients for analysis by immunohistochemistry. Explanted diseased liver was obtained from patients undergoing liver transplantation for liver diseases, including primary sclerosing cholangitis, primary biliary cholangitis, alcoholic liver disease, and non alcoholic steatohepatitis; lymphocytes, hepatocytes, and biliary epithelial cells were isolated as described.<sup>(19–23)</sup>

### STUDY DESIGN

The UK-AIH Consortium immunophenotyping study reported here was set up as a prospective assessment comparing the changes in a set of predefined immune cell subset features for consecutive treatment-naïve patients recruited between May 2015 and November 2016 and evaluating these features in relation to changes in a set of predefined biochemical factors that indicate liver function (alanine aminotransferase [ALT], bilirubin, and immunoglobulin G [IgG]). Immune features compared included the frequencies of nine predefined, innate, and adaptive immune cell subsets (CD4, CD8, CD4CD8, and CD4-CD8-double-negative CD3<sup>+</sup> T cells; CD127<sup>+</sup>CD25<sup>+</sup> CD4 Tregs; CD56<sup>+</sup> NK T cells (NKT); CD19<sup>+</sup> B cells; CD56 bright NK [NK<sup>bright</sup>] and CD56 dim NK [NK<sup>dim</sup>] cells) and their expression of four surface markers (cysteine-X-cysteine receptor 3 [CXCR3], interleukin [IL]-6R, CD161, programmed death ligand 1 [PD1]). The initial body of data indicated important changes in NK and Treg subsets; thus, additional analyses were initiated for subsequent patients to also compare the effector phenotypes of NK subsets and the frequencies and phenotypes of effector and memory populations of CD4, CD8 T cells, and Treg populations before and after steroid therapy.

### ISOLATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS AND IMMUNE PHENOTYPING BY MULTICOLOR FLOW CYTOMETRY

Please see the [Supporting Methods](#).

### FLOW CYTOMETRY ANALYSIS OF LECTIN-LIKE TRANSCRIPT-1 EXPRESSION BY LIVER-INFILTRATING IMMUNE CELLS

Please see the [Supporting Methods](#).

### IMMUNOHISTOCHEMISTRY STAINING OF CHEMOKINE (C-X-C MOTIF) LIGAND-10, FOXP3, AND CD56 IN THE HUMAN LIVER

Pretreatment AIH liver biopsies were stained with anti-chemokine (C-X-C motif) ligand 10 (CXCL-10) (6D4; R&D Systems) and either anti-FOXP3 (236A/E7;



Abcam) or anti-CD56 (CD564; Leica) on the Bond Rx system by using Bond Polymer Refine 3,3'-diaminobenzidine tetrahydrochloride hydrate and Bond Polymer Refine red detection substrates (Novocastra).

## ENZYME-LINKED IMMUNOSORBENT ASSAY OF CXCL-10 SECRETION BY STIMULATED PRIMARY HUMAN HEPATOCYTES AND BILIARY EPITHELIAL CELLS

CXCL-10 concentrations in culture supernatants were measured using a bead-based Bio-Plex Pro assay (Bio-Rad) according to the manufacturer's instructions.

## NK AND T-CELL COCULTURE ASSAYS

Please see the [Supporting Methods](#).

## CLINICAL DATA COLLECTION

ALT, bilirubin, and IgG data were collected for each patient before and 4 months after immunosuppressive therapy. All patients on the study received steroid therapy. There were differences in the specific formulation of steroid given depending on the local practice and whether the patient had cirrhosis (prednisolone) or not (budesonide). Steroid dosage followed national and international guidelines.

## STATISTICAL ANALYSIS

Comparisons between baseline and 4 months were performed using Wilcoxon tests. Changes between baseline and 4 months were compared to the changes in ALT, bilirubin, and IgG using Spearman's correlation coefficients. Comparisons across cell subsets or conditions of experiment were performed using Mann-Whitney, Wilcoxon, or Friedman tests, with Dunn's post-hoc analysis. Analyses were performed using SPSS 22 (IBM Corp., Armonk, NY) or GraphPad Prism software version 6 (San Diego, CA). Data were summarized graphically with error bars representing mean  $\pm$  SEM.  $P < 0.05$  was considered significant.

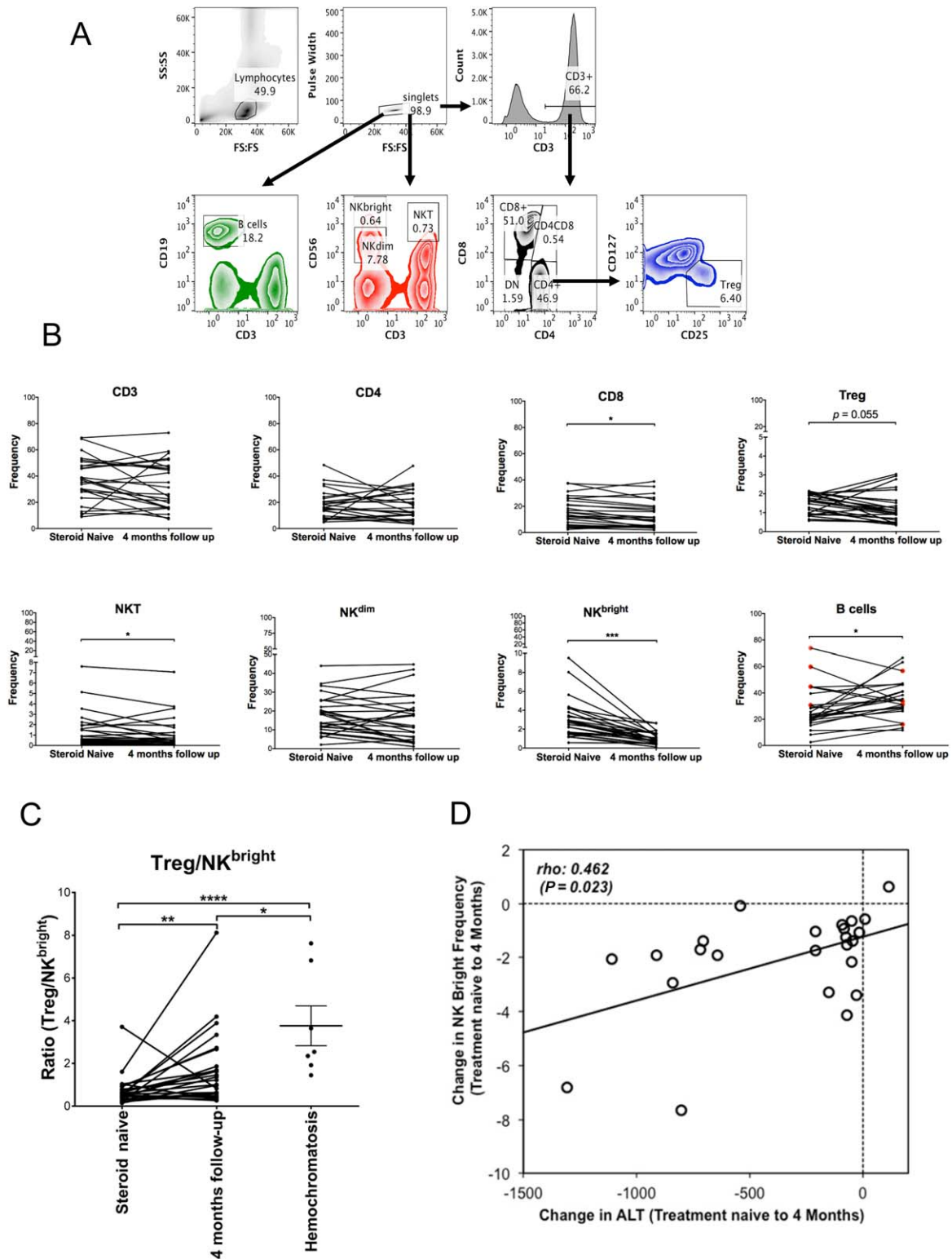
## Results

We studied the baseline and month 4 peripheral immunophenotype in 26 patients (3 male, 23 female;

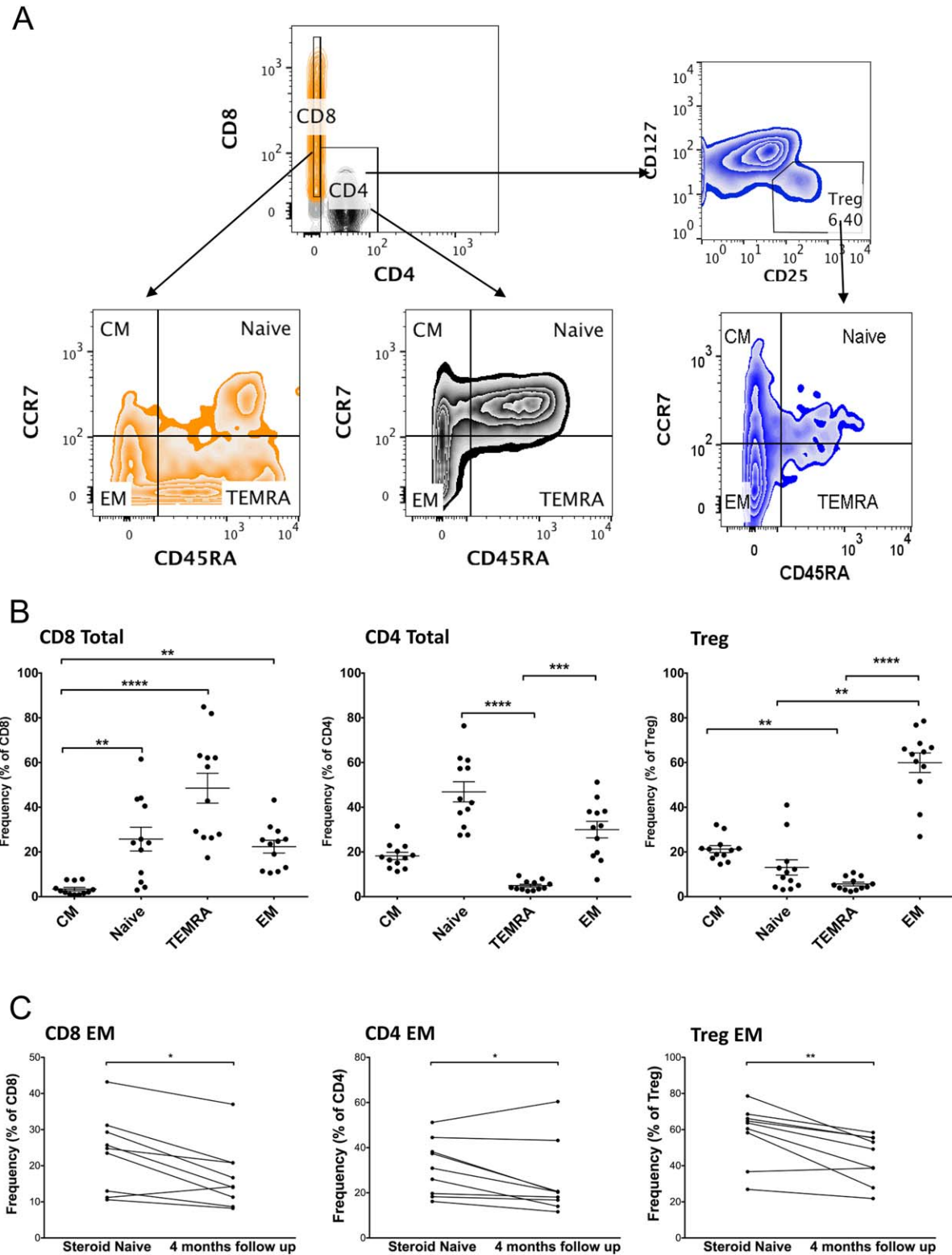
median age 56 years, range 44–66 years) with serologically and histologically confirmed diagnosis of acute AIH from different National Health Service hospitals across the United Kingdom. These patients had clinical markers of disease activity with presenting ALT (369 IU/L, range 82–1,352), bilirubin (26  $\mu$ mol/L, range 10–121), and IgG (23 mg/dL, range 14–38). The detailed clinical characterizations of the patients reported in the study and the medications they received are given in [Supporting Table S1](#).

## NK CELL FREQUENCIES ARE HIGHER IN PATIENTS WITH CORTICOSTEROID-NAIVE AIH AND DECLINE WITH THERAPY

To investigate changes in circulating immune cells in the 26 patients with acute AIH, we compared their peripheral blood immune cell profiles presented at diagnosis (treatment naive/baseline) and after 4 months on corticosteroid therapy. Subsets were defined by the gating strategy in Fig. 1A. Strikingly, frequencies of NK<sup>bright</sup> cells were significantly higher in 25/26 patients at baseline and declined with therapy (median, 2.7% versus 0.9%;  $P < 0.001$ ). Within the total lymphocyte pool, proportions of CD8 T cells (median, 14.4% versus 9.7%;  $P = 0.004$ ; 20/26 patients), NKT (median, 0.63% versus 0.45%;  $P = 0.024$ ; 19/26 patients), and Tregs (median, 1.6% versus 1.0%;  $P = 0.055$ ; 19/26 patients) were also reduced with therapy (Fig. 1B). B-cell frequencies were significantly lower at baseline (median, 23% versus 33%;  $P = 0.03$ ), with increases after therapy observed in 80% of cases (20/25). Other cell subsets did not change significantly (Fig. 1A). Overall, the relative Treg/NK<sup>bright</sup> balance increased significantly with therapy (Fig. 1C), and compared to control patients with hemochromatosis, the Treg/NK<sup>bright</sup> ratio was significantly reduced in treatment-naive patients ( $P < 0.0001$ ). This was not only due to lower NK<sup>bright</sup> frequencies in hemochromatosis but also due to higher Treg frequencies in controls compared to patients with AIH ([Supporting Fig. S2](#)). Furthermore, there was a significant correlation between the changes in ALT and frequencies of NK<sup>bright</sup> cells from baseline to 4-month follow-up ( $P = 0.02$ ) (Fig. 1D; [Supporting Table S2](#)). None of the other effector populations (CD8 and NKT) that declined with therapy altered significantly in their balance with Tregs ([Supporting Fig. S3](#)) or correlated with biochemical markers of disease activity ([Supporting Table S2](#)).



**FIG. 1.** Peripheral blood immune cell frequencies in patients with AIH before and after 4 months immunosuppression therapy. (A) Gating strategy for definition of immune cell subsets, including CD4, CD8, CD4<sup>pos</sup>CD8<sup>neg</sup> (double-negative) T cells; CD4<sup>pos</sup>CD25<sup>pos</sup>CD127<sup>neg</sup> regulatory T cells; CD56<sup>pos</sup> T cells; NKT; CD19<sup>+</sup> B cells; CD56<sup>bright</sup> NK cells (NK<sup>bright</sup>), and CD56<sup>dim</sup> NK cells (NK<sup>dim</sup>). (B) Cell subset frequencies as a proportion of total lymphocytes at baseline and after 4 months. Wilcoxon test, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.0001$ . Red dots indicate patients with high starting B cell frequency that declined dramatically with therapy. (C) Ratio of Tregs to NK<sup>bright</sup> cells in steroid-naive, 4-month follow-up, and hemochromatosis (control) blood. Wilcoxon test, \*\* $P < 0.01$ ; Mann-Whitney U test, \* $P < 0.05$ , \*\*\*\* $P < 0.0001$ . Error bars are means  $\pm$  SEM. (D) Scatterplot and Spearman's correlation coefficient for the association between changes in ALT levels and NK<sup>bright</sup> cell frequencies between baseline and 4 months. Trend line calculated by linear regression is shown.



**FIG. 2.** Frequencies of memory/naive CD8, CD4, and Treg subsets in treatment-naive AIH before and after 4 months immunosuppression. (A) T-cell memory and naive subsets were identified based on expression of CD45RA and CCR7. Representative fluorescence-activated cell sorting plots illustrating the gating strategy are shown. (B) Frequencies before treatment. Friedman test with Dunn's post-hoc analysis,  $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$ ,  $****P < 0.0001$ . (C) Frequencies of EM CD4, CD8, and Tregs before and after 4 months immunosuppression. Wilcoxon test,  $*P < 0.05$ . Abbreviations: CM, central memory; TEMRA, terminally differentiated tissue resident effector memory RA-positive.

Corticosteroid therapy was thus associated with a change in the immune balance toward a higher proportion of Tregs over NK<sup>bright</sup> cells.

## **BOTH REGULATORY MEMORY AND EFFECTOR MEMORY T-CELL FREQUENCIES ARE SIGNIFICANTLY HIGHER IN THE CIRCULATION IN TREATMENT-NAIVE PATIENTS WITH AIH AND DECLINE AFTER THERAPY**

We additionally explored whether T cells had been exposed to antigen by assessing the memory/naive status of the cells in a subgroup of the cohort ( $n = 12$  treatment naive;  $n = 9$  follow-up). We defined memory and naive CD4, CD8, and Treg populations by their expression of CD45RA and chemokine (C-C motif) receptor 7 (CCR7)<sup>(24,25)</sup> as central memory (CD45RA<sup>neg</sup>CCR7<sup>pos</sup>), effector memory (EM; CD45RA<sup>neg</sup>CCR7<sup>neg</sup>), naive (CD45RA<sup>pos</sup>CCR7<sup>pos</sup>), and tissue-resident terminally differentiated effector memory RA<sup>pos</sup> (TEMRA; CD45RA<sup>pos</sup>CCR7<sup>neg</sup>) (Fig. 2A). Within the CD4 and CD8 populations in the treatment-naive state, naive CD4 T-cell and TEMRA CD8 T-cell subsets predominated (Fig. 2B). In contrast, the EM Treg subset was the significantly predominant Treg population (Fig. 2B). In all T-cell classes, we observed significantly higher frequencies of EM cells at baseline compared to follow-up (Fig. 2C).

## **MEMORY Tregs EXPRESS SUPPRESSIVE FUNCTIONAL MARKERS BUT ALSO PRESENT AN EXHAUSTED PHENOTYPE IN PATIENTS WITH CORTICOSTEROID-NAIVE AIH**

FOXP3 transcription factor controls Treg function.<sup>(8)</sup> Cytotoxic T lymphocyte antigen 4 (CTLA-4) leads to transendocytosis of CD80/86 on dendritic cells to down-regulate antigen presentation,<sup>(26)</sup> and CD39 generates immunosuppressive adenosine.<sup>(27)</sup> Memory Tregs had significantly higher expression of these markers compared to other Treg groups in the treatment-naive patients (Fig. 3A,D; Supporting Fig. S4).

When we assessed the proportions of the Treg fractions defined by CD25 and CD45RA expression in treatment-naive patients, we observed a predominance of fraction III (Fig. 3 B,C). However, fraction II had

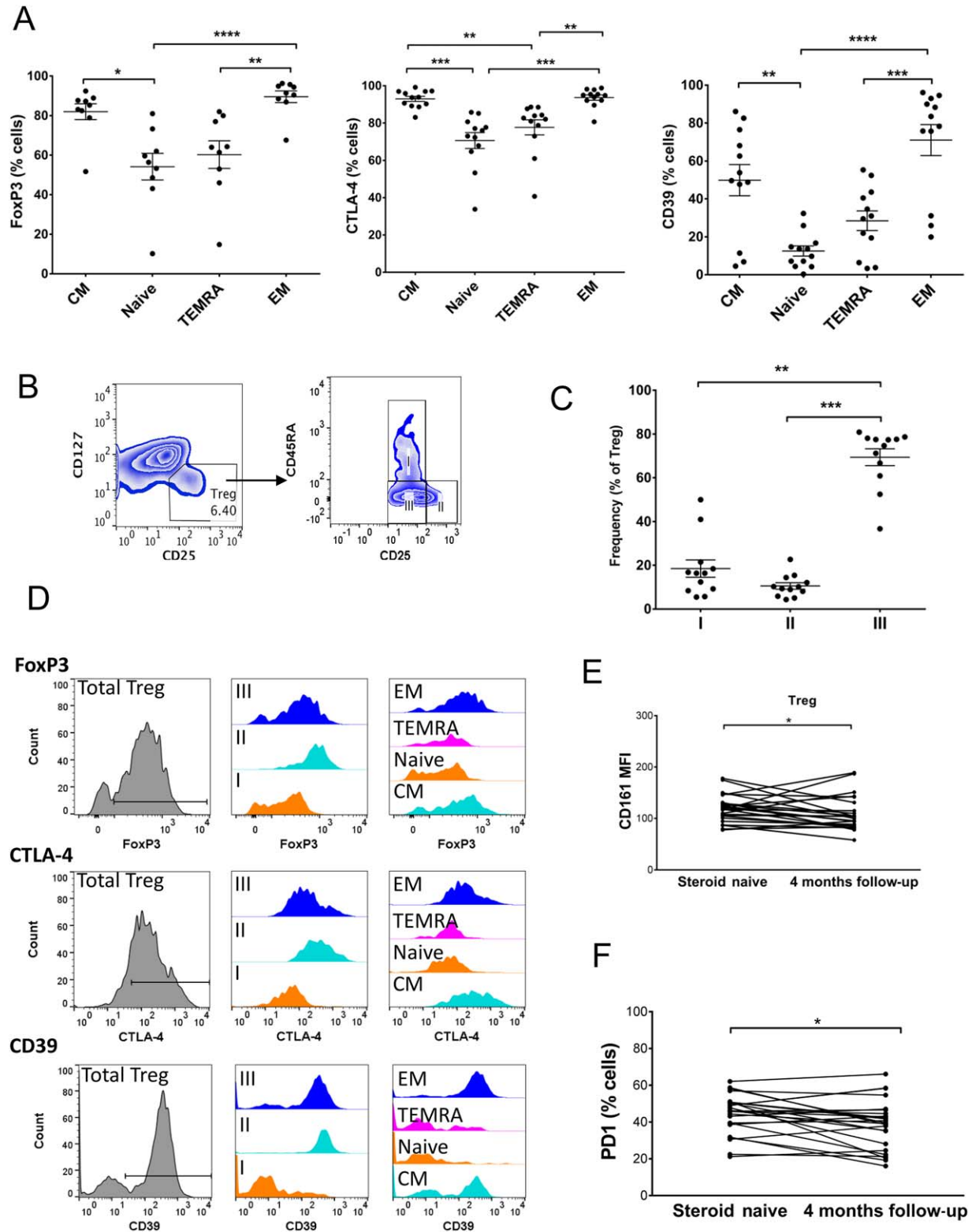
the highest level of Treg functional markers FOXP3, CTLA-4, and CD39 (Fig. 3D; Supporting Fig. S3). Previous studies suggested that fraction III Tregs express CD161 and have a high propensity for differentiation toward a T helper (Th)17 phenotype.<sup>(25,28,29)</sup> We found that CD161 expression on Tregs (median frequency, 12.9% versus 11.0%;  $P = 0.042$ ; median fluorescence intensity, 118 versus 100;  $P = 0.037$ ) was significantly higher before treatment (Fig. 3E). The frequency of CD161<sup>pos</sup> Tregs was also higher before therapy compared to the 4-month follow-up (Supporting Fig. S5). Memory Tregs and fraction III Tregs had the highest expression of CD161 (Supporting Fig. S6A). In addition, PD1, a marker of lymphocyte exhaustion, was reduced on Tregs after 4 months of immunosuppressive therapy (median, 46 versus 40;  $P = 0.027$ ) (Fig. 3F).

## **NK CELL CD161 EXPRESSION LEVELS ARE SIGNIFICANTLY HIGHER IN THE TREATMENT-NAIVE STATE, AND CD161<sup>pos</sup> NK CELLS ARE CHARACTERIZED BY HIGH EXPRESSION OF GRANZYME B AND PERFORIN**

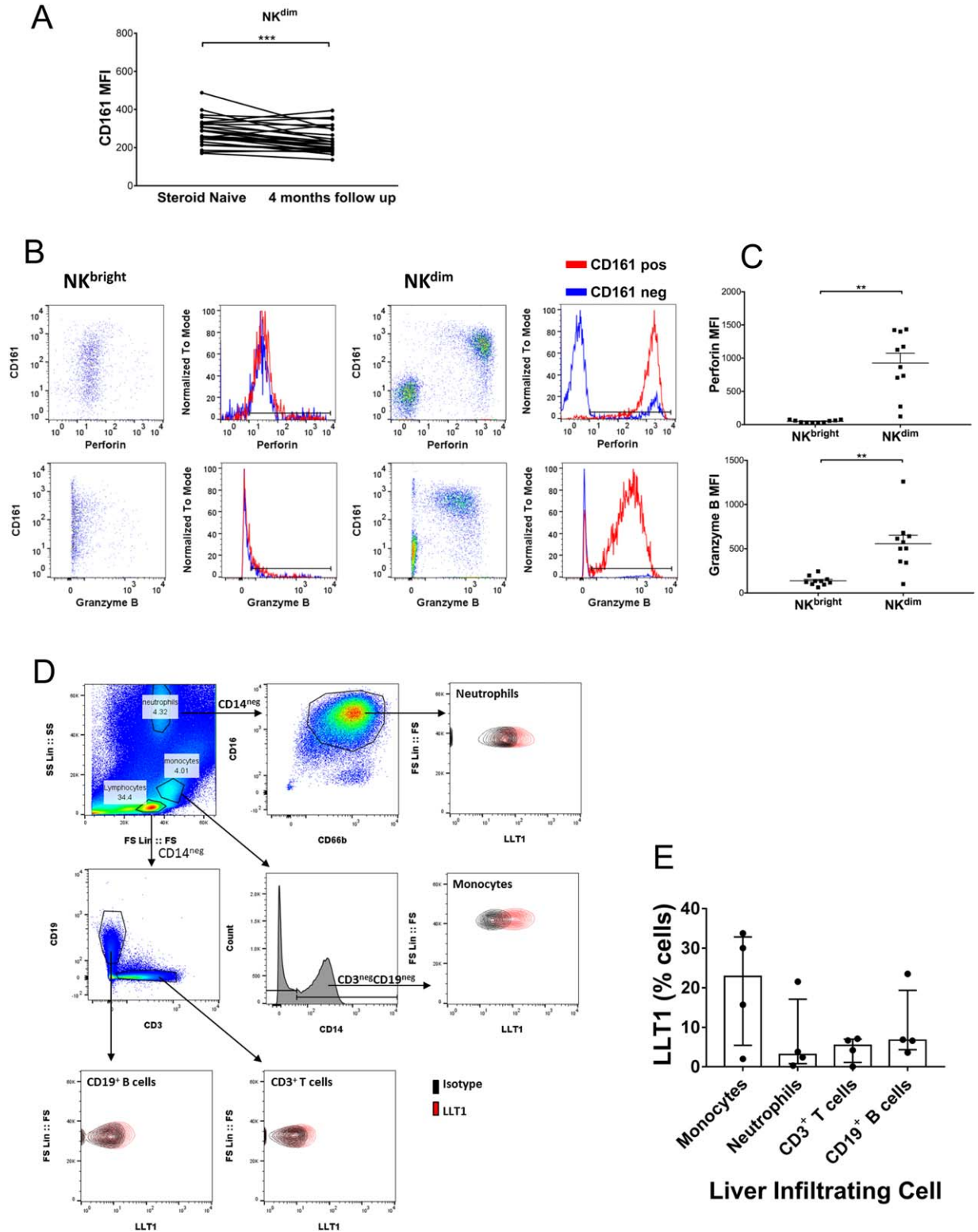
With the exception of CD19<sup>pos</sup> B cells, all immune subsets contained CD161<sup>pos</sup> cells (Supporting Fig. S5). The level of CD161 expression on NK<sup>dim</sup> cells (median fluorescence intensity, 274 versus 217;  $P < 0.001$ ) was significantly higher before treatment compared with after 4 months corticosteroid (Fig. 4A; Supporting Fig. S5). We characterized the expression of functional cytolytic molecules by NK subsets subdivided according to CD161 expression and observed the highest frequencies of cytotoxic factors granzyme B and perforin in the CD161<sup>pos</sup> NK populations. CD161<sup>pos</sup> NK<sup>dim</sup> cells had significantly greater expression of both perforin and granzyme B on a cell by cell basis than NK<sup>bright</sup> cells (Fig. 4B,C; Supporting Fig. S6B).

Because we detected high frequencies of CD161<sup>pos</sup> cells, we explored the expression of the CD161 ligand lectin-like transcript 1 (LLT1), by liver-infiltrating immune cells. We analyzed its expression by cells isolated from explanted autoimmune or inflammatory diseased liver tissue by flow cytometry (Fig. 4D) and detected LLT1 on T and B cells, monocytes, and neutrophils (Fig. 4E).

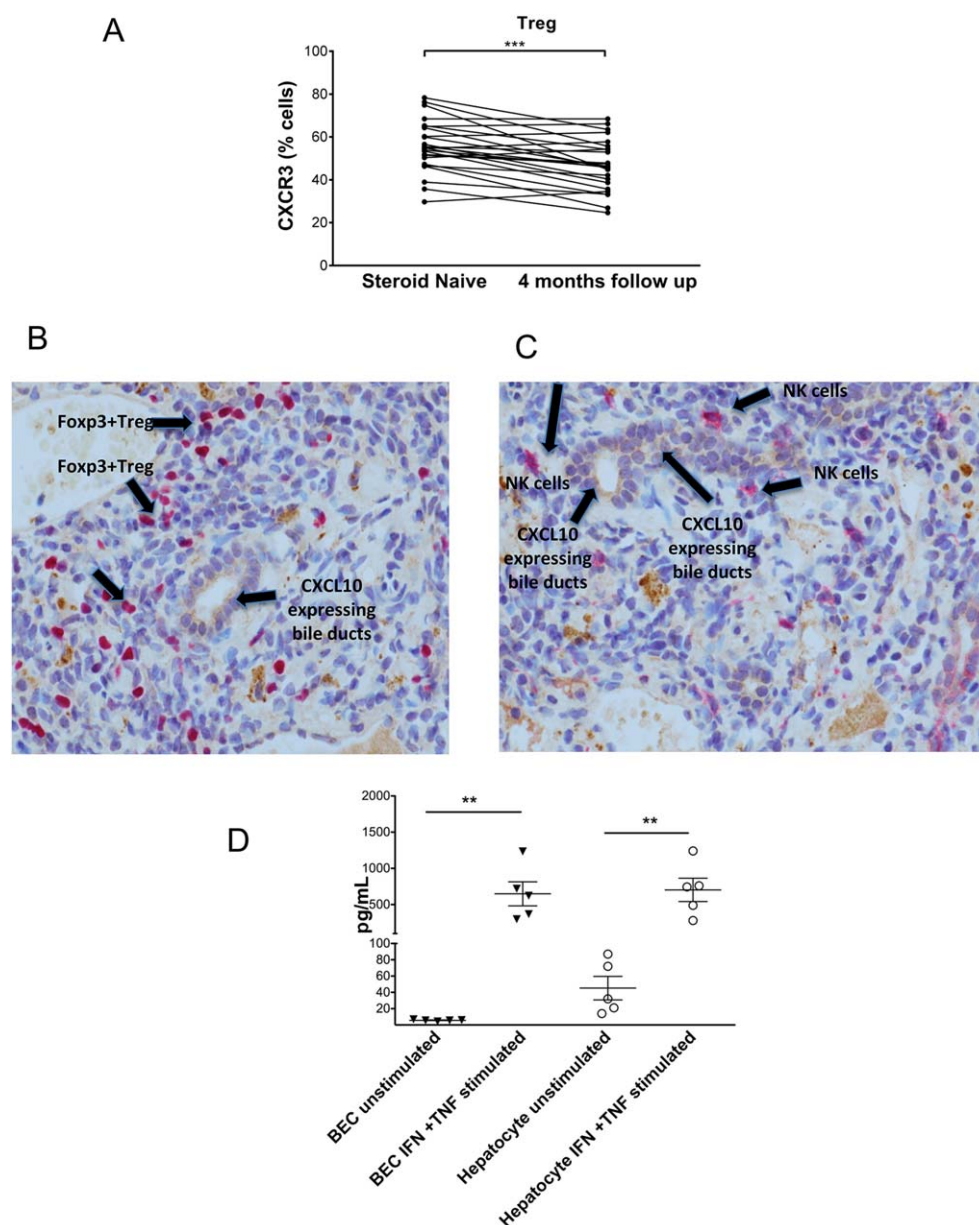




**FIG. 3.** Treg phenotype in acute AIH. (A) Expression of Treg functional proteins FOXP3, CTLA-4, and CD39 by memory/naive Treg subsets defined as in Fig. 2 by CD45RA and CCR7 expression. (B) Gating strategy for Treg fractions based on CD45RA and CD25 expression. (C) Frequencies of fractions in AIH pretreatment. In A and C, tests were Friedman with Dunn's post-hoc analysis,  $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$ ,  $****P < 0.0001$ . (D) Representative flow cytometry overlays showing expression of Treg functional proteins FOXP3, CTLA-4, and CD39 by the total Treg population; fractions I, II, and III Tregs; and the memory and naive Treg subsets. (E) CD161 (MFI) and (F) PD1 (frequency) expression by total Tregs at baseline and after 4 months therapy. Wilcoxon test,  $*P < 0.05$ . Abbreviations: CM, central memory; MFI, median fluorescence intensity; TEMRA, terminally differentiated tissue resident effector memory RA-positive.



**FIG. 4.** Characterization of CD161-expressing NK cells and CD161 ligand LLT1 in AIH. (A) CD161 expression on NK<sup>dim</sup> cells at baseline and after 4 months therapy. Wilcoxon test,  $*P < 0.05$ . (B) Representative flow cytometry plots and overlays showing granzyme B and perforin expression by CD161<sup>pos</sup> and CD161<sup>neg</sup> NK cell populations pretreatment. (C) Summary data on granzyme B and perforin expression by CD161<sup>pos</sup> populations of NK<sup>bright</sup> and NK<sup>dim</sup> cells pretreatment;  $**P < 0.01$ . (D) Flow cytometry gating strategy for identification of immune cell subsets, including neutrophils, monocytes, T cells, and B cells, among isolated liver-infiltrating immune cells and representative staining of LLT1 versus isotype control on these populations. (E) Summary data on LLT1 expression by liver-infiltrating immune cells isolated from primary biliary cirrhosis, primary sclerosing cholangitis, alcoholic liver disease, and nonalcoholic steatohepatitis explanted livers. Abbreviation: MFI, median fluorescence intensity.



**FIG. 5.** CXCL-10 distribution in treatment-naive AIH livers colocalizes with CD56<sup>+</sup> NK cells and FOXP3<sup>+</sup> Tregs. (A) CXCR3 expression by total Tregs at baseline and after 4 months therapy. Wilcoxon test, \* $P < 0.05$ . (B,C) Dual staining of CXCL-10 and either (B) CD56 or (C) FOXP3 on liver biopsy sections taken at diagnosis of AIH (CXCL-10, brown staining; FOXP3<sup>+</sup> Tregs, CD56<sup>+</sup> NK cells, red staining). (D) CXCL-10 concentrations in culture supernatants prepared from untreated and IFN $\gamma$  + TNF $\alpha$ -treated hepatocytes and biliary epithelial cells. Data are mean  $\pm$  SEM. Abbreviation: BEC, biliary epithelial cell.

## Tregs AND NK CELLS IN TREATMENT-NAIVE AIH SHOW HIGH EXPRESSION OF CXCR3, AND CXCR3 LIGAND CXCL-10 DERIVES FROM INFLAMED LIVER HEPATOCYTES AND BILIARY CELLS

CXCR3 was expressed by all subsets of immune cells, but expression levels varied (Supporting Fig. S7). CXCR3<sup>pos</sup> Treg frequencies in the total Treg

population were significantly higher in the treatment-naive state than at follow-up ( $P < 0.001$ ) (Fig. 5A). The expression of CXCR3 was highest in the memory compared to naive Treg populations ( $P < 0.05$ ) (Supporting Fig. S8). We have previously reported a role for CXCR3 in lymphocyte homing to the liver,<sup>(2,17,30)</sup> but to confirm its potential involvement in the pathogenesis of acute AIH, we stained pretreatment liver biopsies for the CXCR3-binding chemokine CXCL-10. CXCL-10 was detected on bile ducts in the portal tracts and was closely associated with infiltrating



FOXP3<sup>pos</sup> Treg cells (Fig. 5B) and CD56<sup>pos</sup> NK cells (Fig. 5C). Furthermore, we demonstrated that CXCL-10 was secreted by human hepatocytes and biliary epithelial cells in response to inflammatory cytokines *in vitro* (Fig. 5D).

## REGULATORY T-CELL CONTROL OF NK CELLS

To investigate whether the significant increase in the ratio of NK cells to Tregs could have functional implications in AIH pathogenesis, we tested whether activated NK cell effector responses could be controlled by Tregs *in vitro*. CD3<sup>neg</sup>CD56<sup>pos</sup> NK cells activated overnight with cytokines IL-12 and IL-15 secreted high levels of IFN $\gamma$  but expressed little TNF $\alpha$  or CD107a (Fig. 6A). In agreement with the idea that there is inadequate Treg control over NK in the AIH disease process, we observed a very clear trend of suppression of NK activity by Tregs ( $P = 0.0625$ ); marked suppression by Tregs occurred in five out of the six experiments performed. This was in contrast to the effects of coculture of NK cells with activated non-Treg effectors, which led instead to significantly elevated IFN $\gamma$  expression by NK ( $P = 0.0312$ ) (Fig. 6B).

## IMMUNOLOGICAL MARKERS AND PERIPHERAL BLOOD LIVER BIOCHEMISTRY AND IgG LEVEL

Finally, we explored whether any phenotypes examined in this study could define patients with treatment-naïve AIH who are nonresponders to corticosteroid therapy. We defined subjects as nonresponders based on their having  $1.5 \times$  upper limit of normal (ULN) values for ALT,  $1.5 \times$  ULN values for bilirubin, and  $1.5 \times$  ULN values for IgG at 4 months. Only 2 patients fulfilled these criteria, so any interpretation is limited; however, these patients were characterized by high levels of CXCR3 on their NK<sup>bright</sup> cells and CD4 T cells (Fig. 6C,D) and lower expression of the exhaustion marker PD1 on CD4 T cells (Fig. 6E).

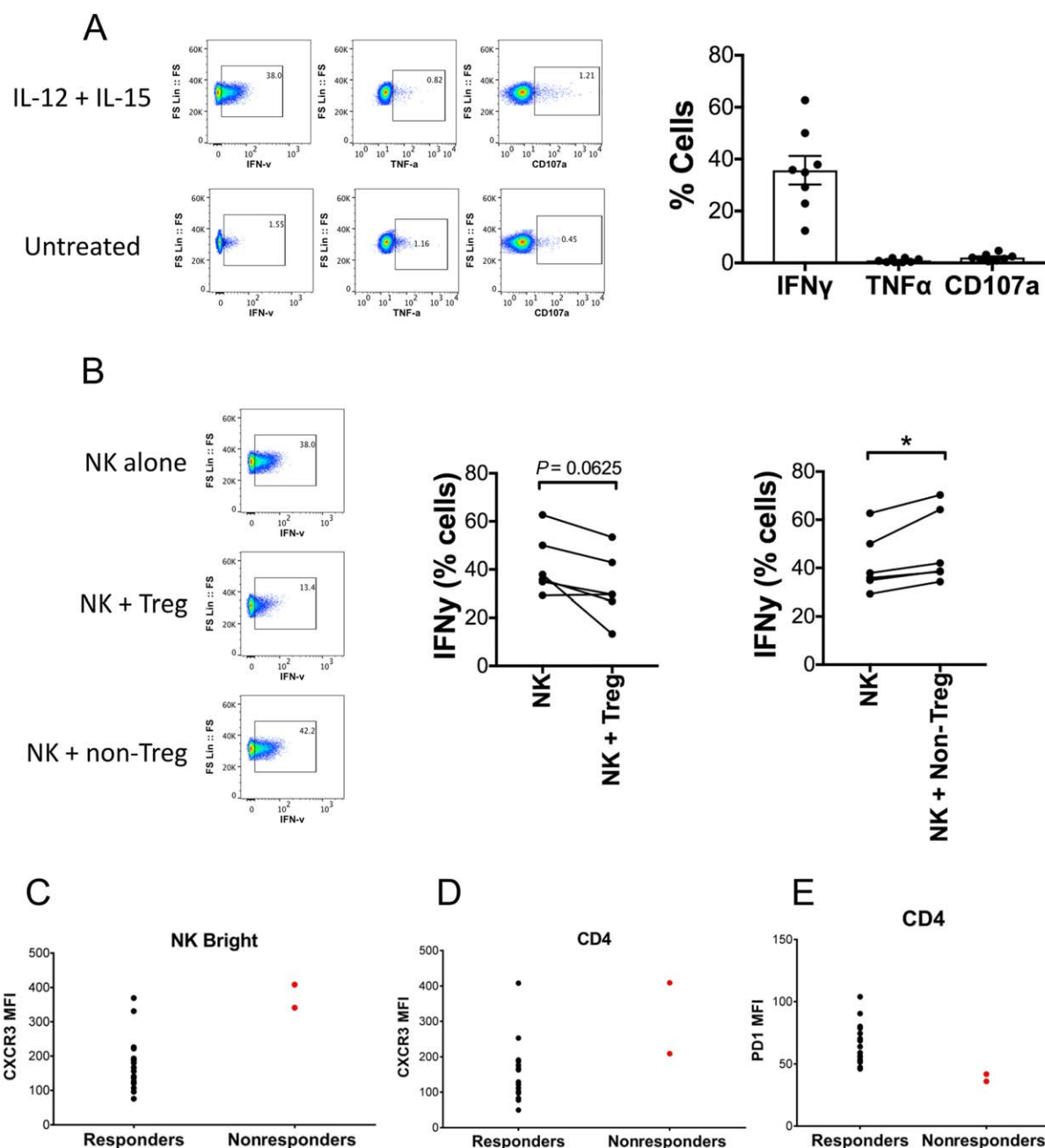
## Discussion

Type-1 AIH is an immune-mediated liver disease that usually responds to immunosuppression. Because patients are promptly treated with corticosteroids, it is

difficult to study patients in the treatment-naïve state before immunosuppression is started. Our study is one of the first to characterize in detail the changes in immune cell composition before and after treatment in patients with AIH. The study was not designed to report the alterations in total cell numbers with therapy but rather to discuss the influence of therapy on the ratios of the different innate and adaptive immune subsets and to describe the changes in their individual phenotypes; as such, this study offers important insight into the subsets whose relative precedence may be key to AIH pathogenesis and thus crucial to target in future targeted therapies.

The two main findings of this study are a significant elevation of activated effector CD56<sup>bright</sup> NK cells in the blood prior to therapy and a concomitant increase in regulatory T cells. In the healthy state, NK cells constitute 5%-15% of peripheral blood cells and are abundant in the liver.<sup>(31)</sup> NK cells have been implicated in liver injury<sup>(32)</sup> and in the pathogenesis of autoimmune diseases, such as systemic lupus erythematosus and diabetes.<sup>(33,34)</sup> They provide an early host defense to infection or injury and have the ability to kill other cells rapidly by cytolysis through their release of cytolytic mediators without prior stimulation.<sup>(35)</sup> We observed a significant increase in CD56<sup>bright</sup> NK cells in untreated patients with AIH (25/26 cases) that fell after 4 months of immunosuppressive treatment. CD56<sup>bright</sup> cells are found in the liver but under normal circumstances are present at low frequencies in the blood. They are critical for local innate immune responses and are increased at sites of inflammation in autoimmune diseases.<sup>(36)</sup> We also observed a higher frequency of NK<sup>dim</sup> cells (up to 40%) in treatment-naïve patients. It is possible that in treatment-naïve AIH, NK cells enter the peripheral circulation from the liver, but perhaps more likely, they are released into the blood from lymphoid tissues where they are found at high frequencies. In parallel to an increase in NK cell frequency, we also observed an increased Treg frequency in the circulation of patients with treatment-naïve AIH. Tregs are crucial to maintain peripheral immune tolerance and control effector immune cells.<sup>(37,38)</sup> The increase in the Treg population may thus be an attempt to suppress immune activation stimulated by the causative autoantigen in acute AIH. Although both NK<sup>bright</sup> and Treg cell frequencies were increased in treatment-naïve patients, the Treg versus NK<sup>bright</sup> cell ratio was reduced, suggesting a skewing of the immune balance toward the effector arm in the treatment-naïve state. Importantly, Tregs





**FIG. 6.** Regulation of NK cell function by activated regulatory and nonregulatory T cells. (A,B) NK cells, Tregs, and non-Tregs were isolated from peripheral blood. NK cell degranulation (CD107a), IFN $\gamma$ , and TNF $\alpha$  production, with overnight IL-12 (20 ng/mL) + IL-15 (20 ng/mL) stimulation monitored by flow cytometry in the presence or absence of activated Tregs or non-Tregs. (A) Representative and summary data on NK degranulation and cytokine production with stimulation. (B) IFN $\gamma$  production by NK cells cultured overnight alone or 1:1 with either Tregs or non-Tregs under IL-12 (20 ng/mL) + IL-15 (20 ng/mL) stimulation. (C-E) Expression of immune cell surface markers in responders and nonresponders after 4 months corticosteroid treatment. CXCR3 MFI on (C) NK<sup>bright</sup> cells and (D) CD4 T cells. (E) PD1 MFI on CD4 T cells. Abbreviation: MFI, median fluorescence intensity.

were weak suppressors of NK cell production of inflammatory cytokines; thus, the data suggest that loss of NK cell regulation could underlie hepatic damage in

AIH. In support of this conclusion, the NK<sup>bright</sup> cell frequency correlated with levels of ALT in treatment-naïve patients.

A previous *in vivo* study<sup>(39)</sup> in diabetes suggested that Tregs suppress NK function by depleting IL-2, which is critical for NK cell survival and optimal function, from the local environment. Although in the present study the suppression of NK cell production of inflammatory cytokines did not reach significance, there was a clear indication that Tregs offer a level of regulation over activated NK cells in the normal state. It is possible that in the treatment-naïve state of AIH, Tregs may be too exhausted, a conclusion supported by their elevated PD1 expression pretreatment, or too infrequent to effectively control NK cell activity. Overall, the data suggest that loss of NK cell regulation involving inadequate Treg-mediated control could underlie hepatic damage in AIH. In support of this conclusion, the NK<sup>bright</sup> cell frequency correlated with levels of ALT in treatment-naïve patients.

The changes in peripheral blood described in this study are informative, although it is likely that the most important responses pathologically are those that occur within the liver. Our data suggest that the expanded peripheral populations we have seen have the potential to be recruited to the inflamed liver because they express high levels of the chemokine receptor CXCR3, which facilitates the recruitment of effector and regulatory T cells to the inflamed human liver<sup>(2,30)</sup> through interactions with its ligand CXCL-10.<sup>(2,17,30)</sup> We detected both CXCR3<sup>pos</sup> Tregs and NK cells residing close to CXCL-10-expressing bile ducts in liver biopsies from patients with acute AIH.

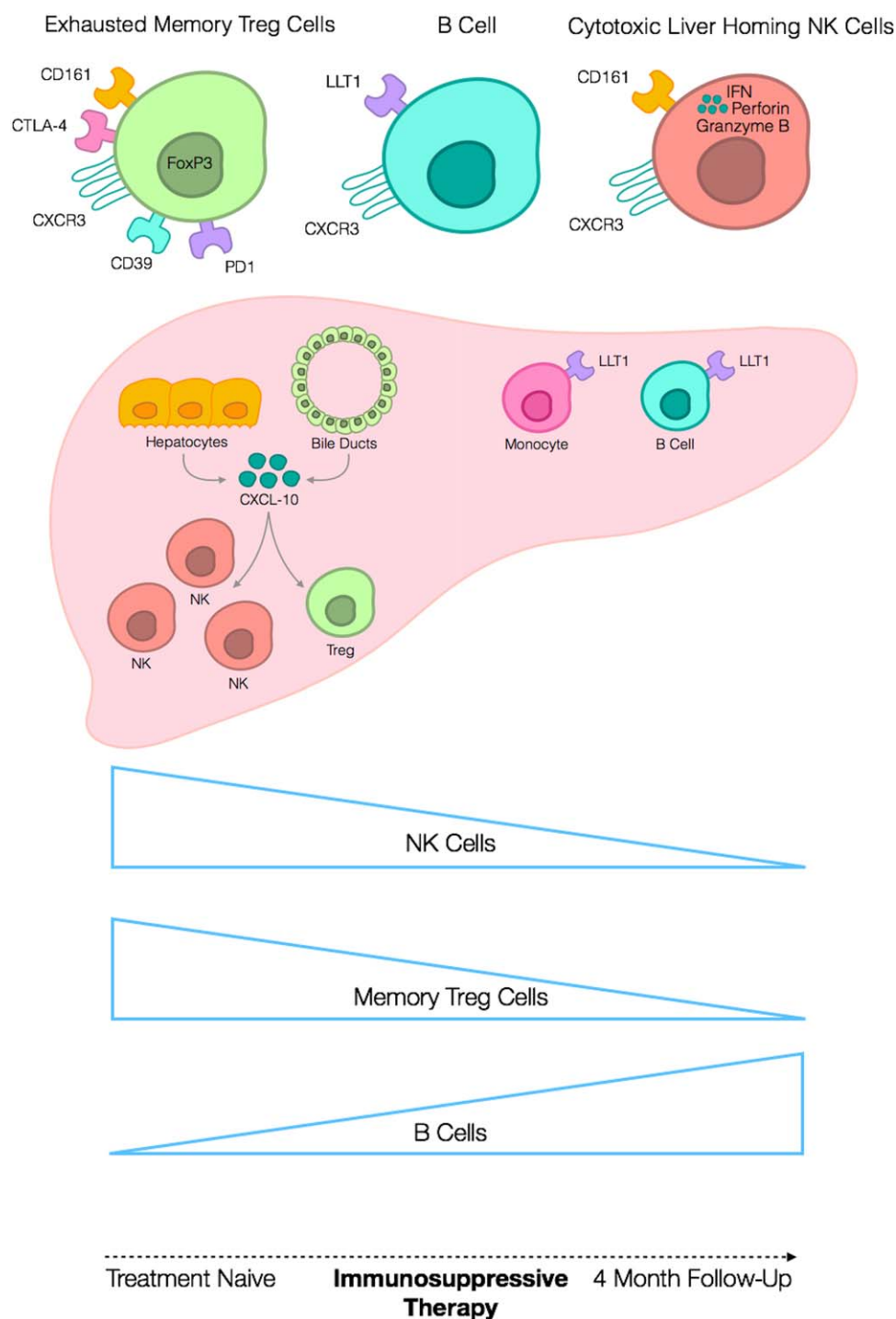
We examined the Treg subset in treatment-naïve patients in more detail and found that these cells were predominantly CD45RA<sup>neg</sup> memory, which suggests that they have been exposed to antigen. Specifically, Tregs and also CD4 and CD8 cells were predominantly of an EM phenotype before exposure to immunosuppression, and this proportion declined significantly in all subsets after therapy. Thus, it is likely that the EM component of Tregs is reflective of extensive efforts to suppress antigen-primed effector T cells in the liver.<sup>(40)</sup> Future studies into the T-cell receptor repertoire of both CD4 and Tregs in treatment-naïve AIH would be of interest to determine whether these cells are clonally expanded subsets that respond to the same antigen. In addition, Treg functional markers FOXP3, CD39, and CTLA-4 were highest on the memory population, suggesting that these are suppressive memory Tregs. However, these cells also expressed high levels of PD1, which has been associated with T-cell exhaustion, suggesting that these cells may not be fully functional. This could

explain in part why immune-mediated liver injury progresses in spite of increased Treg frequencies.

Treg cells in peripheral blood can be divided into three fractions,<sup>(25)</sup> with fraction III Tregs characterized by the expression of CD161,<sup>(28,29)</sup> which has been described as a potentially plastic Treg fraction that can change its lineage toward Th17.<sup>(28,29)</sup> CD161 is a lectin-like receptor expressed on human NK cells and T lymphocytes.<sup>(41)</sup> We have previously demonstrated that CD161<sup>pos</sup> T cells in the human liver include both Th17 and Tc17 cells<sup>(42,43)</sup> and that these cells are implicated not only in liver injury but also in the pathogenesis of autoimmune Crohn's disease.<sup>(44)</sup> LLT1, a ligand for CD161,<sup>(45)</sup> is present on antigen-presenting cells, germinal center B cells, and Kupffer cells.<sup>(46,47)</sup> Interactions between CD161 on NK cells and LLT1 on target cells inhibit NK cell-mediated cytotoxicity and cytokine production.<sup>(46-49)</sup> We observed significantly higher CD161 expression on both NK cells and Tregs in treatment-naïve AIH that declined after therapy and demonstrated that LLT1 is expressed on liver-infiltrating monocytes, B cells, and neutrophils. Thus, CD161 could be activated by ligand-bearing cells in the inflamed liver to down-regulate effector responses; but if insufficient ligand is present, this feedback could be overwhelmed.

We noted that patients who did not respond to immunosuppressive therapy had a reduced exhausted T-cell phenotype with lower PD1 expression, which agrees with a lack of control over effector cells in these patients with therapy. The B cell frequency was elevated in 20% (5/25 cases) of treatment-naïve patients, and this group might be one that would respond to anti-B cell therapy, such as rituximab.<sup>(39,50)</sup>

Our study has its limitations. As only small volumes of blood (5-10 mL) were supplied by each participating hospital, the full volume was taken to isolate the peripheral blood mononuclear cells for immune phenotyping; count data on each subset in the whole blood were not collected. CD45 selection and dead cell exclusion were not included in the phenotyping panel. This decision was taken in the study design in order to maximize the depth of immune subset phenotyping with the small volume of sample available and access to only nine color instruments; it was also based on knowledge that CD45 frequencies of >99% and dead cell frequencies of 1%-3% are consistently observed with peripheral blood samples processed under the same conditions and are seen to have negligible impact on the frequencies of the populations of interest in the study (Supporting Figs. S9 and S10; Supporting



**FIG. 7.** Diagrammatic illustration of the immune cell composition changes occurring in treatment-naïve AIH with 4 months corticosteroid therapy. Regulatory T cells in the peripheral blood of patients with treatment-naïve acute AIH are predominantly effector memory in phenotype ( $CD45RA^{neg}CCR7^{neg}$ ) and express functional proteins CD39, CTLA-4, and FOXP3. They also express the liver-homing receptor CXCR3 and exhaustion marker PD1. B cells and monocytes in inflamed AIH livers express the CD161 ligand LLT1, and CD161 expression by both NK cells and Tregs is elevated before therapy in patients with acute AIH. CXCL-10 secreted by inflamed hepatocytes and bile ducts attracts CXCR3-expressing NK cells and Tregs to the site of hepatitis in AIH. Both NK cells and memory Tregs are expanded significantly in the peripheral immune cell composite of patients with acute AIH before the start of therapy compared to after 4 months on corticosteroid. In contrast, the B cell frequency is significantly lower at the treatment-naïve acute stage and recovers with corticosteroid.

Tables S3 and S4). There were differences in the steroid regimen used by different contributing centers, and these might have influenced the magnitude of phenotypic change observed. However, an increase in variability would be more reasonable to expect; as such, the conclusions of disease-relevant phenotypes made in this study are believed to be sound and relevant. Limitation of sample volume to under 10 mL with the current UK-AIH ethics meant that *in vitro* assays to test the possibility that a lack of NK cell regulation by Tregs contributes to AIH pathogenesis could not be evaluated on patient blood as such analysis required around 50 mL. Despite these limitations, the study has identified interesting and novel phenotypic changes with therapy in a unique, rare, and poorly understood cohort, which can now guide the direction of further studies to address in more detail the pathologic mechanisms underlying AIH pathogenesis and direct improved and stratified therapeutic strategies with reduced side effects.

In conclusion, our study is the first to report longitudinal changes in detailed leucocyte subsets in AIH (Fig. 7). The changes in the balance of NK cells and Tregs in treatment-naïve patients suggest a switch in the effector/regulatory balance that might be amenable to therapy. The relevance of the NK cell changes is supported by the correlation with liver injury early in disease. Further studies using deep immunophenotyping with mass cytometry in well-characterized cohorts of patients are required before immunophenotyping can be used for treatment stratification.

**Acknowledgment:** We are indebted to all the local principle investigators and members of the clinical and research teams who have recruited to UK-AIH treatment-naïve patients for this study.

## REFERENCES

- 1) Kerr JF, Cooksley WG, Searle J, Halliday JW, Halliday WJ, Holder L, et al. The nature of piecemeal necrosis in chronic active hepatitis. *Lancet* 1979;2:827-828.
- 2) Oo YH, Weston CJ, Lalor PF, Curbishley SM, Withers DR, Reynolds GM, et al. Distinct roles for CCR4 and CXCR3 in the recruitment and positioning of regulatory T cells in the inflamed human liver. *J Immunol* 2010;184:2886-2898.
- 3) Wong LL, Kendrick S, Dyson JK, Jones D. Understanding the unmet need in autoimmune hepatitis, The United Kingdom Autoimmune Hepatitis Study (UK-AIH). *Hepatology* 2016;64:815A.
- 4) Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 1995;155:1151-1164.
- 5) Peiseler M, Sebode M, Franke B, Wortmann F, Schwinge D, Quaas A, et al. FOXP3+ regulatory T cells in autoimmune hepatitis are fully functional and not reduced in frequency. *J Hepatol* 2012;57:125-132.
- 6) Taubert R, Hardtke-Wolenski M, Noyan F, Wilms A, Baumann AK, Schlue J, et al. Intrahepatic regulatory T cells in autoimmune hepatitis are associated with treatment response and depleted with current therapies. *J Hepatol* 2014;61:1106-1114.
- 7) Jeffery HC, Jeffery LE, Lutz P, Corrigan M, Webb GJ, Hirschfield GM, et al. Low-dose interleukin-2 promotes STAT5 phosphorylation, Treg survival and CTLA-4-dependent function in autoimmune liver diseases. *Clin Exp Immunol* 2017;188:394-411.
- 8) Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 2003;299:1057-1061.
- 9) Bennett CL, Christie J, Ramsdell F, Brunkow ME, Ferguson PJ, Whitesell L, et al. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat Genet* 2001;27:20-21.
- 10) Schepis D, Gunnarsson I, Eloranta ML, Lampa J, Jacobson SH, Karre K, et al. Increased proportion of CD56bright natural killer cells in active and inactive systemic lupus erythematosus. *Immunology* 2009;126:140-146.
- 11) Pridgeon C, Lennon GP, Pazmany L, Thompson RN, Christmas SE, Moots RJ. Natural killer cells in the synovial fluid of rheumatoid arthritis patients exhibit a CD56bright, CD94 bright, CD158negative phenotype. *Rheumatology (Oxford)* 2003;42:870-878.
- 12) Racanelli V, Rehermann B. The liver as an immunological organ. *Hepatology* 2006;43(Suppl. 1):S54-S62.
- 13) Werner JM, Heller T, Gordon AM, Sheets A, Sherker AH, Kessler E, et al. Innate immune responses in hepatitis C virus-exposed healthcare workers who do not develop acute infection. *Hepatology* 2013;58:1621-1631.
- 14) Gerosa F, Baldani-Guerra B, Nisii C, Marchesini V, Carra G, Trinchieri G. Reciprocal activating interaction between natural killer cells and dendritic cells. *J Exp Med* 2002;195:327-333.
- 15) Schuch A, Hoh A, Thimme R. The role of natural killer cells and CD8(+) T cells in hepatitis B virus infection. *Front Immunol* 2014;5:258.
- 16) Maini MK, Peppas D. NK cells: a double-edged sword in chronic hepatitis B virus infection. *Front Immunol* 2013;4:57.
- 17) Oo YH, Banz V, Kavanagh D, Liaskou E, Withers DR, Humphreys E, et al. CXCR3-dependent recruitment and CCR6-mediated positioning of Th-17 cells in the inflamed liver. *J Hepatol* 2012;57:1044-1051.
- 18) Hennes EM, Zeniya M, Czaja AJ, Pares A, Dalekos GN, Krawitt EL, et al.; International Autoimmune Hepatitis Group. Simplified criteria for the diagnosis of autoimmune hepatitis. *Hepatology* 2008;48:169-176.
- 19) Bhogal RH, Hodson J, Bartlett DC, Weston CJ, Curbishley SM, Haughton E, et al. Isolation of primary human hepatocytes from normal and diseased liver tissue: a one hundred liver experience. *PLoS One* 2011;6:e18222.
- 20) Chen YY, Jeffery HC, Hunter S, Bhogal R, Birtwistle J, Braitch MK, et al. Human intrahepatic regulatory T cells are functional,



- require IL-2 from effector cells for survival and are susceptible to Fas ligand-mediated apoptosis. *Hepatology* 2016;64:138-150.
- 21) Holt AP, Haughton EL, Lalor PF, Filer A, Buckley CD, Adams DH. Liver myofibroblasts regulate infiltration and positioning of lymphocytes in human liver. *Gastroenterology* 2009;136:705-714.
  - 22) Shetty S, Bruns T, Weston CJ, Stamatakis Z, Oo YH, Long HM, et al. Recruitment mechanisms of primary and malignant B cells to the human liver. *Hepatology* 2012;56:1521-1531.
  - 23) Lalor PF, Edwards S, McNab G, Salmi M, Jalkanen S, Adams DH. Vascular adhesion protein-1 mediates adhesion and transmigration of lymphocytes on human hepatic endothelial cells. *J Immunol* 2002;169:983-992.
  - 24) Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 1999;401:708-712.
  - 25) Miyara M, Yoshioka Y, Kitoh A, Shima T, Wing K, Niwa A, et al. Functional delineation and differentiation dynamics of human CD4<sup>+</sup> T cells expressing the FoxP3 transcription factor. *Immunity* 2009;30:899-911.
  - 26) Qureshi OS, Zheng Y, Nakamura K, Attridge K, Manzotti C, Schmidt EM, et al. Trans-endocytosis of CD80 and CD86: a molecular basis for the cell-extrinsic function of CTLA-4. *Science* 2011;332:600-603.
  - 27) Deaglio S, Dwyer KM, Gao W, Friedman D, Ucheva A, Erat A, et al. Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *J Exp Med* 2007;204:1257-1265.
  - 28) Afzali B, Mitchell PJ, Edozie FC, Povolieri GA, Dowson SE, Demandt L, et al. CD161 expression characterizes a subpopulation of human regulatory T cells that produces IL-17 in a STAT3-dependent manner. *Eur J Immunol* 2013;43:2043-2054.
  - 29) Pesenacker AM, Bending D, Ursu S, Wu Q, Nistala K, Wedderburn LR. CD161 defines the subset of FoxP3<sup>+</sup> T cells capable of producing proinflammatory cytokines. *Blood* 2013;121:2647-2658.
  - 30) Curbishley SM, Eksteen B, Gladue RP, Lalor P, Adams DH. CXCR3 activation promotes lymphocyte transendothelial migration across human hepatic endothelium under fluid flow. *Am J Pathol* 2005;167:887-899.
  - 31) Ajuebor MN, Wondimu Z, Hogaboam CM, Le T, Proudfoot AE, Swain MG. CCR5 deficiency drives enhanced natural killer cell trafficking to and activation within the liver in murine T cell-mediated hepatitis. *Am J Pathol* 2007;170:1975-1988.
  - 32) Vermijlen D, Luo D, Froelich CJ, Medema JP, Kummer JA, Willems E, et al. Hepatic natural killer cells exclusively kill splenic/blood natural killer-resistant tumor cells by the perforin/granzyme pathway. *J Leukoc Biol* 2002;72:668-676.
  - 33) Hou Y, Zhang C, Xu D, Sun H. Association of killer cell immunoglobulin-like receptor and human leucocyte antigen-Cw gene combinations with systemic lupus erythematosus. *Clin Exp Immunol* 2015;180:250-254.
  - 34) Dotta F, Censini S, van Halteren AG, Marselli L, Masini M, Dionisi S, et al. Coxsackie B4 virus infection of beta cells and natural killer cell insulinitis in recent-onset type 1 diabetic patients. *Proc Natl Acad Sci U S A* 2007;104:5115-5120.
  - 35) Meier UC, Owen RE, Taylor E, Worth A, Naoumov N, Willberg C, et al. Shared alterations in NK cell frequency, phenotype, and function in chronic human immunodeficiency virus and hepatitis C virus infections. *J Virol* 2005;79:12365-12374.
  - 36) Gross CC, Schulte-Mecklenbeck A, Runzi A, Kuhlmann T, Posevitz-Fejfar A, Schwab N, et al. Impaired NK-mediated regulation of T-cell activity in multiple sclerosis is reconstituted by IL-2 receptor modulation. *Proc Natl Acad Sci U S A* 2016;113:E2973-2982.
  - 37) Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. *Cell* 2008;133:775-787.
  - 38) Jeffery HC, Braitch MK, Brown S, Oo YH. Clinical potential of regulatory T cell therapy in liver diseases: an overview and current perspectives. *Front Immunol* 2016;7:334.
  - 39) Sitrin J, Ring A, Garcia KC, Benoist C, Mathis D. Regulatory T cells control NK cells in an insulinitis lesion by depriving them of IL-2. *J Exp Med* 2013;210:1153-1165.
  - 40) Gantner F, Leist M, Lohse AW, Germann PG, Tiegs G. Concanavalin A-induced T-cell-mediated hepatic injury in mice: the role of tumor necrosis factor. *Hepatology* 1995;21:190-198.
  - 41) Fergusson JR, Smith KE, Fleming VM, Rajoriya N, Newell EW, Simmons R, et al. CD161 defines a transcriptional and functional phenotype across distinct human T cell lineages. *Cell reports* 2014;9:1075-1088.
  - 42) Kang YH, Seigel B, Bengsch B, Fleming VM, Billerbeck E, Simmons R, et al. CD161(+)CD4(+) T cells are enriched in the liver during chronic hepatitis and associated with co-secretion of IL-22 and IFN-gamma. *Front Immunol* 2012;3:346.
  - 43) Billerbeck E, Kang YH, Walker L, Lockstone H, Grafmueller S, Fleming V, et al. Analysis of CD161 expression on human CD8<sup>+</sup> T cells defines a distinct functional subset with tissue-homing properties. *Proc Natl Acad Sci U S A* 2010;107:3006-3011.
  - 44) Kleinschek MA, Boniface K, Sadekova S, Grein J, Murphy EE, Turner SP, et al. Circulating and gut-resident human Th17 cells express CD161 and promote intestinal inflammation. *J Exp Med* 2009;206:525-534.
  - 45) Llibre A, Klenerman P, Willberg CB. Multi-functional lectin-like transcript-1: a new player in human immune regulation. *Immunol Lett* 2016;177:62-69.
  - 46) Llibre A, Lopez-Macias C, Marafioti T, Mehta H, Partridge A, Kanzig C, et al. LIT1 and CD161 expression in human germinal centers promotes B cell activation and CXCR4 downregulation. *J Immunol* 2016;196:2085-2094.
  - 47) Rosen DB, Cao W, Avery DT, Tangye SG, Liu YJ, Houchins JP, et al. Functional consequences of interactions between human NKR-P1A and its ligand LIT1 expressed on activated dendritic cells and B cells. *J Immunol* 2008;180:6508-6517.
  - 48) Chalan P, Bijzet J, Huitema MG, Kroesen BJ, Brouwer E, Boots AM. Expression of lectin-like transcript 1, the ligand for CD161, in rheumatoid arthritis. *PLoS One* 2015;10:e0132436.
  - 49) Llibre A, Garner L, Partridge A, Freeman GJ, Klenerman P, Willberg CB. Expression of lectin-like transcript-1 in human tissues. *F1000Res* 2016;5:2929.
  - 50) Burak KW, Swain MG, Santodomingo-Garzon T, Lee SS, Urbanski SJ, Aspinall AI, et al. Rituximab for the treatment of patients with autoimmune hepatitis who are refractory or intolerant to standard therapy. *Can J Gastroenterol* 2013;27:273-280.

Author names in bold designate shared co-first authorship.

## Supporting Information

Additional Supporting Information may be found at [onlinelibrary.wiley.com/doi/10.1002/hep4.1163/full](http://onlinelibrary.wiley.com/doi/10.1002/hep4.1163/full).